Supporting information

Modulation of the Lifespan of *C. elegans* by Controlled Release of Nitric Oxide

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Experimental section

Materials

Dichloromethane (DCM) was dried over calcium hydride and distilled before use. Methoxy poly(ethylene glycol) (PEG-OH, M_n = 2000) was azeotropically dried by toluene. Ethanolamine, 2-methoxyethylamine, succinic anhydride, 4',6-diamidino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium bicarbonate, ethanol, acetic acid, hydrochloric acid, 4-bromo-1,8-naphthalic anhydride, dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), sodium nitrite, *N,N'*-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopridine (DMAP), deuterated chloroform ($CDCl_3$) and dimethyl sulfoxide-*d*6 (*d*-DMSO) were purchased from Aladdin and used as received. Cell counting Kit-8 (CCK-8) and Calcein-AM were purchased from Beyotime Biotechnology, China. CellROX Deep Red Reagent was purchased from Invitrogen (Carlsbad, CA). 2-Phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) was purchased from Sigma-Aldrich. The reaction between PTIO and NO was shown in **Scheme S1**. All reagents were of analytical grade and used directly as received.

Characterization

^{1}H NMR spectra and ^{13}C NMR spectra were recorded on a BRUKER AV400 Spectrophotometer (400M Hz) with $CDCl_3$ or *d*-DMSO as solvent and tetramethylsilane (TMS) as an internal reference. EI-HRMS and ESI-MS spectra were acquired on a Micromass GCT spectrometer and a TOF mass spectrometer respectively. Gel permeation chromatography (GPC) was measured on a Waters 1515, and the samples were dissolved in anhydrous THF with polystyrene as an internal reference and THF as eluent. UV-vis spectra were obtained on a UV-2450 UV-visible spectrophotometer. Fluorescence spectra were obtained on an F-4500 fluorescence spectrophotometer at room temperature. Dynamic light scattering (DLS) measurements were conducted with a BECKMAN COULTER Delasa Nano C particle analyzer. Transmission electron microscopy (TEM) images were recorded on a JEOL JEM1400 electron microscope with accelerating voltage of 100 KV. Confocal laser scanning microscopy (CLSM) was performed on a Nikon A1R. MTT assay was conducted with a spectrophotometric microplate reader (Thermo Multiskan MK3 spectrometer) at a wavelength of 560 nm.

Synthesis of BNA-OH

4-Bromo-1,8-naphthalic anhydride (20 g, 72.18 mmol) and ethanolamine (4.4 g, 72.18 mmol) were added in 500 mL ethanol. Under reflux condition, the mixture was stirred for 8 h, and then was filtered and washed by ethanol to afford a crude product, which was purified by recrystallization in co-solvent of ethanol and water to obtain BNA-OH (19.6 g) in an 85 % yield. ^{1}H NMR (400 MHz, d-DMSO), δ ppm: 8.56 (t, 2H), 8.34 (d, 1H), 8.23 (d, 1H), 8.0 (t, 1H), 4.83 (t, 1H), 4.14 (t, 2H), 3.62 (q, 2H).

Synthesis of MOEANA-OH

BNA-OH (10 g, 31.24 mmol), 2-methoxyethylamine (2.58 g, 34.36 mmol) and $NaHCO_3$ (2.89 g, 34.36 mmol) were added into 300 mL DMSO. The mixture was stirred and heated to 110 ℃ for 12 h and then cooled to room temperature. After that, the reaction solvent was evaporated under reduced pressure. The resulting residue was transferred into water, then, extracted by CH_2Cl_2 three times. The organic layer was dried with Na_2SO_4, filtered and rotary evaporated to obtain the crude product, which was further purified with a silica gel column with CH_2Cl_2 and MeOH (100 : 2, v/v) as the eluent to afford the MOEANA-OH (6.1 g) in an 62 % yield. ^{1}H NMR (400 MHz, $CDCl_3$), δ ppm: 8.53 (d, 1H), 8.42 (d, 1H), 8.12 (d, 1H), 7.59 (t, 1H), 6.68 (d, 1H), 5.78 (s, 1H), 4.44 (t, 2H), 3.97 (q, 2H), 3.78 (t, 2H), 3.57 (q, 2H), 3.48 (s, 3H), 2.89 (t, 1H). EI-HRMS calculated for $C_{17}H_{18}N_2O_4$ [M^+] 314.1267, found 314.1265.

Synthesis of NORM

MOEANA-OH (1 g, 3.18 mmol) was dissolved in a co-solvent of AcOH, HCl, CH_2Cl_2 (10 : 0.5 : 7, v/v) (40 mL) and cooled to 0 °C. Keep stirring, sodium nitrite (329.13 mg, 4.77 mmol) was added into the mixture. After that the ice bath was removed and the reaction mixture was stirred at room temperature for another 2 h. A saturated $NaHCO_3$ solution was added to the flask until the solution pH was adjusted to neutral. The reaction mixture was extracted three times with CH_2Cl_2, then, collected organic layer, dried with Na_2SO_4, filtered and rotary evaporated to obtain the crude product. The crude product was further purified by silica gel column using MeOH and CH_2Cl_2 (1 : 100, v/v) as eluent to afford the NORM (420 mg) with 38 % yield. ^{1}H NMR (400 MHz, $CDCl_3$), δ

ppm: 8.70-8.55 (m, 2H), 8.08 (d, 1H), 7.84 (d, 1H), 7.80-7.70 (m, 1H), 4.46-4.39 (m, 2H), 4.31 (t, 2H), 4.00-3.91 (m, 2H), 3.55 (t, 2H), 3.28 (s, 1H), 3.17 (s, 2H). EI-MS calculated for $C_{17}H_{18}N_3O_5$ [M+H$^+$] 344.1, found 344.1.

Synthesis of PEG-COOH

PEG-OH (2 g, 1 mmol), succinic anhydride (1 g, 10 mmol) and DMAP (244 mg, 2 mmol) were dissolved in CH_2Cl_2. After that, the reaction mixture was stirred constantly with 48 h at room temperature. Afterwards, the reaction solution was poured into 400 mL NaCl solution then extracted by CH_2Cl_2 three times. The organic layer was dried with Na_2SO_4, filtered and rotary evaporated to obtain concentrated solution, which was further purified by precipitation with ether several times to obtain PEG-COOH (1.72 g) in an 86 % yield. ^{1}H NMR (400 MHz, $CDCl_3$), δ ppm: 4.22 (t, 2H), 3.79 (t, 1H), 3.8-3.5 (m, 178H), 3.44 (t, 1H), 3.35 (s, 3H), 2.65-2.55 (m, 4H).

Synthesis of PEG-NORM

Esterification reaction was used to synthesize PEG-NORM. PEG-COOH (300 mg, 0.14 mmol), NORM (500 mg, 1.46 mmol) and DMAP (34 mg, 0.28 mmol) were dissolved in 30 mL DCM in an ice bath. DCC (288 mg, 1.4 mmol) was dissolved in 10 mL DCM and then dropwise added into the solution with argon protection. The ice bath was then removed and the reaction mixture was stirred constantly with another 48 h at room temperature. Afterwards, the reaction solution was poured into 100 mL NaCl solution then extracted by CH_2Cl_2 three times. The organic layer was dried with Na_2SO_4, filtered and rotary evaporated to obtain concentrated solution, which was further purified by precipitation with ether several times to obtain PEG-NORM (280 mg) in an 82 % yield. ^{1}H NMR (400 MHz, $CDCl_3$), δ ppm: 8.86-8.62 (m, 2 H), 8.14 (d, 1H), 7.92-7.74 (m, 2H), 4.56-4.44 (m, 4H), 4.35 (t, 1H), 4.20-4.10 (m, 2H), 3.82 (t, 1H), 3.72-3.44 (m, 183H), 3.38 (s, 3H), 3.32 (s, 1H), 3.20 (s, 2H), 2.66-2.54 (m, 4H).

Preparation the PEG-NORM nanoparticles

PEG-NORM (10 mg) in THF (1 mL) was dropwise added into 4 mL deionized water at room temperature. The solution was dialyzed against deionized water with a dialysis membrane (MWCO = 1000) for 24 h to remove THF.

Detection of the released NO from the PEG-NORM nanoparticles by Griess assay

Griess assay, a typical method to quantitative detect nitric oxide, was used to detect the released NO from the PEG-NORM nanoparticles. The detection principle of the method is that the released NO molecules, after contact with water, could be converted into nitrate or nitrite then react with griess agent and finally converted into an azo dye that could be quantitatively determined using a microplate reader or UV-vis absorption spectroscopy (λ = 540 nm). Before testing, a standard curve was established by $NaNO_2$ standard samples (1 μM - 20 μM) for calculation of NO concentrations. Detailed detection steps are described below. 600 μL griess reagent Ⅰ was added into 600 μL the nanoparticles solution (irradiated with UV light for different durations) and shaken for 1 min. Then, 600 μL griess reagent Ⅱ was added into the mixture solution and shaken for another 2 min at room temperature. At last, the mixture was measured by a UV-visible spectrophotometer.

Detection of the released NO from the PEG-NORM nanoparticles by self-calibration

When the PEG-NORM nanoparticles were irradiated with UV light, the NO was immediately released and corresponding molecules of PEG-MOEANA-OH were also produced. Thus, the fluorescence was turned on. According to the enhancement of fluorescence intensity at 558 nm and standard curve of MOEANA-OH, the released NO can be quickly and accurately calculated. The detailed detection steps are described below. 2 mL solution of the nanoparticles or NORM illuminated with UV light for different durations was measured by a fluorescence spectrophotometer (excitation wavelength: 445nm).

Cell culture

Human lung adenocarcinoma (A549) cells and Human L-02 hepatocytes were kindly provided by the Institute of Biochemistry and Cell Biology, SIBS, Chinese Academy of Sciences, and the passage number was performed within 10 times. A549 and L-02 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS) and antibiotics (50 units per mL penicillin and 50 units per mL streptomycin) under a humidified atmosphere with 5 % CO_2 at 37 °C.

Using self-calibration to detect intracellular NO release from the PEG-NORM

Real time and in situ detecting intracellular NO release can be realized by self-calibration with CLSM. A549 cells with a density of 5×10^5 cells per dish were seeded onto the glass-bottomed dish and cultured with DMEM supplemented with 10% FBS for 24 h. After addition of the PEG-NORM nanoparticles (20 μg mL^{-1}) and incubation for another 24 h, the cells were washed with PBS 3 times to remove residual the nanoparticles. Then the cells were fixed with 4 % paraformaldehyde solution for 30 min and washed with PBS three times. After that the cells were stained with DAPI to mark the nuclei and washed with PBS three times. Then the cells were put on confocal laser scan microscopy (Nikon AIR) and irradiated with UV light for different durations (0 s, 5 s, 10 s, 30 s), meanwhile, the corresponding images were collected. Excitation wavelength is 488 nm, and emission wavelength is 525 nm.

Cell manipulation with PEG-NORM nanoparticles

Dark toxicity: A549 cells were seeded into a 96-well plate at 5000/well and then cultured at 37 ℃ for 24 h. The nanoparticles with different concentrations dispersed in culture medium was added into the wells and incubated for another 24 h. Subsequently, the cells were washed with sterilized PBS three times, and the culture medium was replaced with 200 μL fresh DMEM included 20 μL of MTT (5 mg mL^{-1}). The cells were incubated for another 4 h. After that, 150 μL DMSO was added into the wells with gentle shaking to extract the formazan products. Then, the absorbance was measured at 560 nm using a microplate reader. Cell viability (%) was calculated according to the equation: cell viability (%) = $(OD_{test} - OD_{background})/(OD_{control} - OD_{background}) \times 100$, where OD_{test} is the absorbance in the presence of sample solutions, and $OD_{control}$ is the absorbance of the control group.

Photo-toxicity: A549 cells were seeded into a 96-well plate at 5000/well and then cultured at 37 ℃ for 24 h. The nanoparticles with different concentrations dispersed in culture medium was added into the wells and incubated for another 24 h. Subsequently, the cells were washed with sterilized PBS 3 times to remove residual nanoparticles, and fresh culture medium was added into the plate. Then the cells were irradiated with UV light (80 mW cm^{-2}) for 45s, and put back into incubator for

another 24 h. Finally, cell viability was evaluated using MTT assay.

Cell proliferation assay: The cell proliferation of A549 cells was assayed with CCK-8. A549 cells were seeded into a 96-well plate at 1500/well and then cultured at 37 ℃ for 24 h. The nanoparticles (20 μg mL^{-1}) were added into the wells and incubated for another 24 h. Subsequently, the cells were washed with sterilized PBS 3 times to remove residual nanoparticles, and 200 μL fresh culture medium containing 20 μL CCK-8 solution was added into each plate. Then the cells were irradiated with UV light (80 mW cm^{-2}) for only two seconds, and put back into incubator. 2 d, 4 d, 6 d later, the OD450 values was measured respectively in the absorbance at 450 nm by using a microplate reader. The A549 cells without any treatment were used as control group. This experiment was conducted in triplicate, and the significant difference was also calculated by means of one-way analysis of variance (ANOVA).

CLSM was also utilized to visually evaluate the cell proliferation. A549 cells were seeded into a 6-well plate at 10000/well and then cultured at 37 ℃ for 24 h. The nanoparticles (20 μg mL^{-1}) were added into the wells and incubated for another 24 h. Subsequently, the cells were washed with sterilized PBS 3 times to remove residual nanoparticles, and 200 μL fresh culture medium was added into each plate. Then the cells were irradiated with UV light (80 mW cm^{-2}) for only two seconds, and put back into incubator for 7 d. The A549 cells were stained with Calcein-AM before imaging with a confocal laser scan microscope (Nikon AIR). Without treated A549 cells were used as control group.

Detection intracellular oxidative stress: The CellROX Deep Red probe was used to detect intracellular oxidative stress, which is nonfluorescent while in a reduced state and, upon oxidation, exhibits red fluorescence with excitation/emission maxima at 640/665 nm. A549 cells were seeded onto the glass-bottomed dish with a density of 5×10^5 cells per dish and cultured for 24 h. After addition of the PEG-NORM nanoparticles (20 μg mL^{-1}) and incubation for another 24 h, the cells were washed with PBS 3 times to remove the residual nanoparticles. Then CellROX Deep Red Reagent was added into each dish and incubated for another 30 min. After that, the cells were washed 3 times with PBS and fixed with 4 % paraformaldehyde solution for 30 min. Afterwards, the cells were irradiated with UV light (80 mW cm^{-2}) for different durations (0 s, 2 s, 10 s, 45 s). Confocal laser scanning microscope (Nikon AIR) was used to detect intracellular oxidative stress

and observe intracellular NO release from the PEG-NORM nanoparticles.

Worm strains and culture

Nematodes were wild-type N2 and Escherichia coli (*E. coli*) OP50 were provided by the Caenorhabditis Genetics Center (CGC, University of Minnesota, USA). *C. elegans* were cultured at 20 °C in petri dishes on nematode growth medium (NGM) seeded with *E. coli* OP50. For further experiments, the worms were maintained in the same stage via the synchronization procedure.

Using fluorescence microscope to detect individuals NO release from the PEG-NORM in *C.elegans*

Synchronized worms at the L4 stage of development were exposed to test solution of nanoparticles (10 μg mL^{-1}) for 24 h. After that worms were washed with M9 buffer 3 times to remove residual nanoparticles. And then worms were put on Leica DM4B fluorescence microscope (Leica, Germany) and irradiated with UV light (130 mW cm^{-2}) for different durations (0 s, 5 s, 10 s, 30 s), meanwhile, the corresponding images recorded by screen recording software were collected. Excitation wavelength is 488 nm, and emission wavelength is 525 nm.

Worm Exposure

The toxicity was assessed by means of the endpoints of germline cell apoptosis, the body length, brood size, and life-span assay. For apoptosis, body length and life-span assay, 24 h after hatching, synchronized worms were exposed to test solution of nanoparticles (5 μg mL^{-1}) for 24 h. Subsequently, the worms were washed 3 times with sterilized M9 to remove residual nanoparticles, and 200 μL fresh M9 with *E. coli* OP50 was added into each well. Then the worms were irradiated separately with UV light (130 mW cm^{-2}) for 0 s, 1 s, 3 s, 5 s, 10 s, 30 s, 60 s, and put back into incubator for another 24 h. For brood size assay, the L1 stage hermaphrodites were exposed to test solution of nanoparticles (5 μg mL^{-1}) for 24 h, then the step were same as described above.

Body length assay

Twenty to thirty worms were placed in slide after exposure, the body length of worms were measured by using the Leica DM4B fluorescence microscope (Leica, Germany).

Apoptosis assay

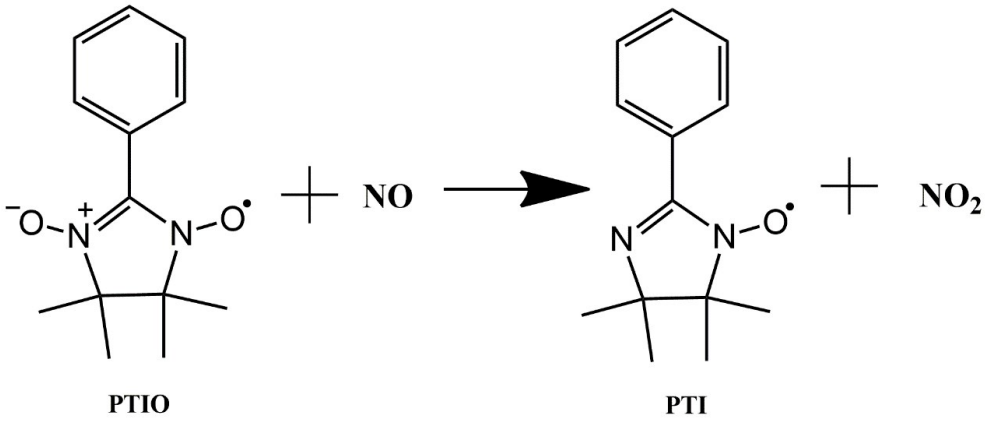
Apoptotic germ cells were measured by acridine orange (AO) vital staining. After exposure, worms were stained with 500 μL of 25 μg/mL AO and incubated at 20 °C for 50 min without light. The worms were transferred to a 30 mm NGM plate with *E. coli* OP50 and allowed to recover for 40 min in the dark. AO staining of positive cell corpses were scored using Leica DM4B fluorescence microscope (Leica, Germany). The apoptotic cells appeared yellow-green after AO staining, representing increased DNA fragmentation, whereas intact cells were uniformly green.

Brood size

After exposure, randomly picked worms were transferred to the 3 mm NGM plates covered with concentrated *E. coli* OP50 and then move to new plates every 24 h until worms stopped laying eggs (approximately 5 d). All eggs were cultured at 20 °C for 24 h, and the newly hatched larvae and eggs were counted.

Life-span assay

Worms were conducted in 96-well plates containing M9 buffer with 5-FudR(20 μg/mL) to prevent offspring generation. E. coli OP50 was added as a food source before irradiated with UV light. All worms were continuously exposed to NO released by nanoparticles until all worms were dead, which was identified by the lack of response to mechanical stimulation.



Scheme S1. The reaction between PTIO and NO.

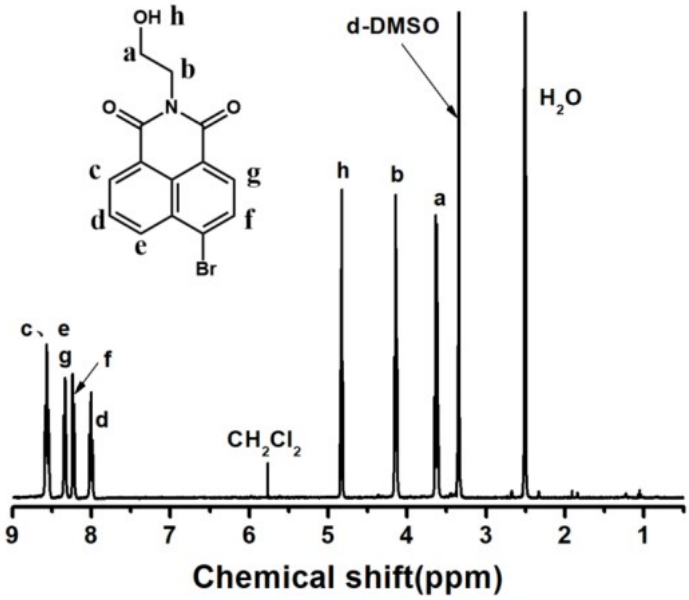


Figure S1. ^{1}H NMR spectrum of BNA-OH

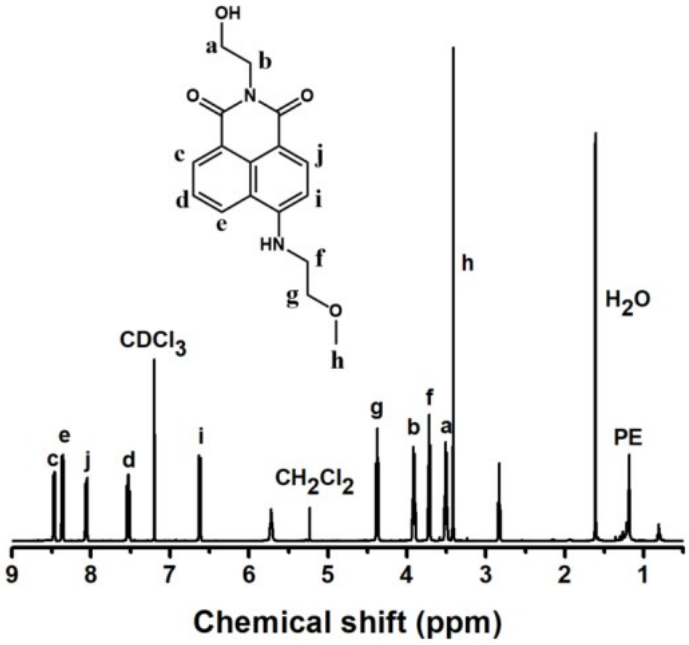


Figure S2. [1]H NMR spectrum of MOEANA-OH

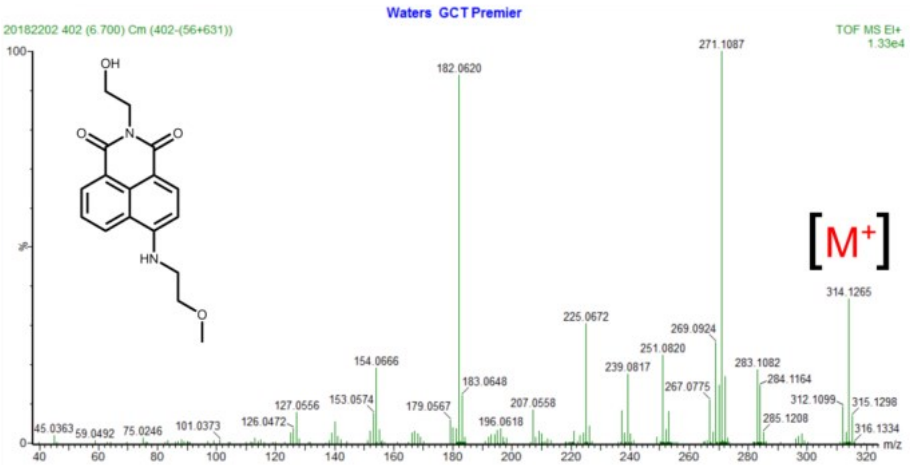


Figure S3. Mass spectrum of MOEANA-OH

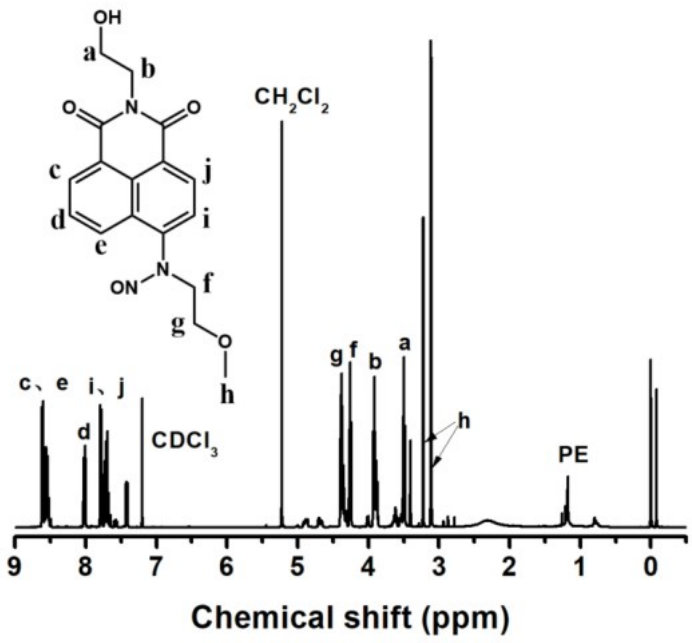


Figure S4. ^{1}H NMR spectrum of NORM

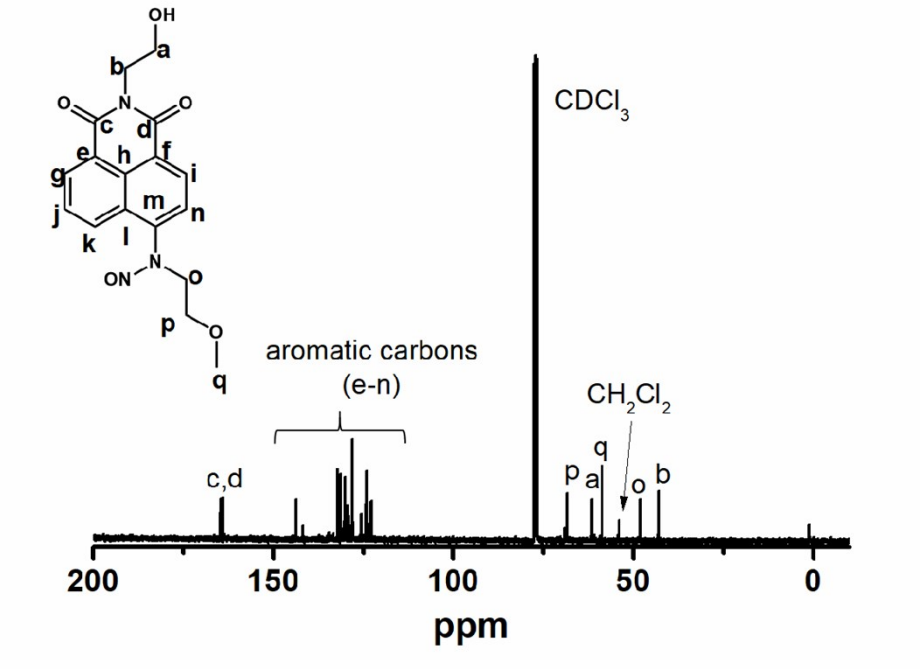


Figure S5. ^{13}C NMR spectrum of NORM

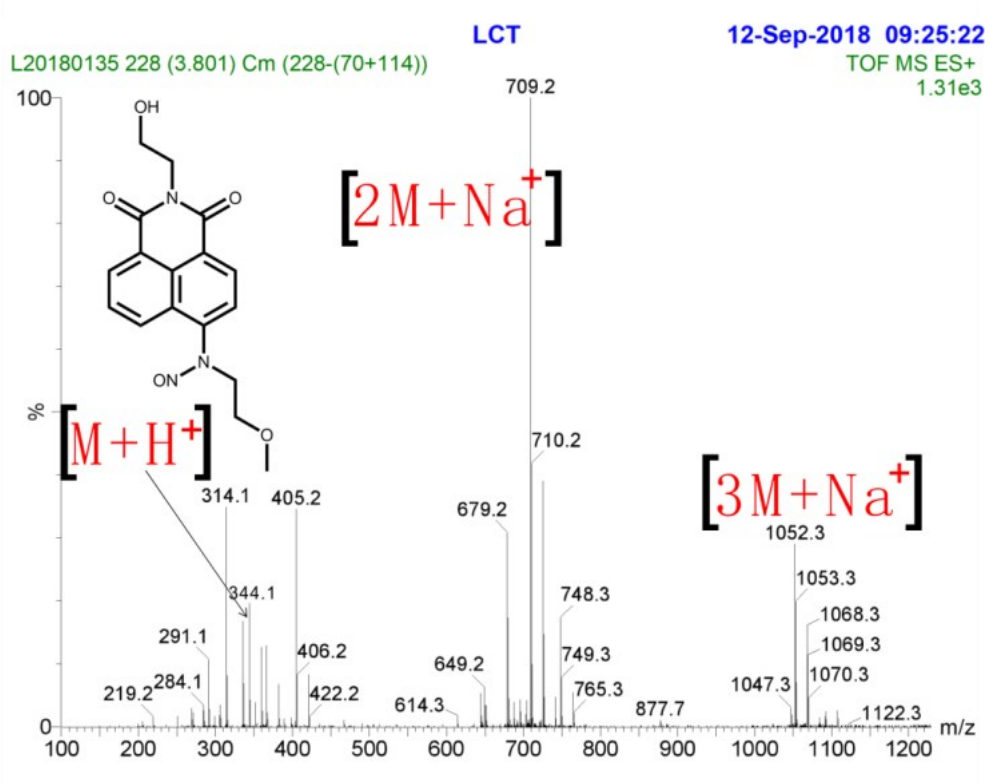


Figure S6. Mass spectrum of NORM

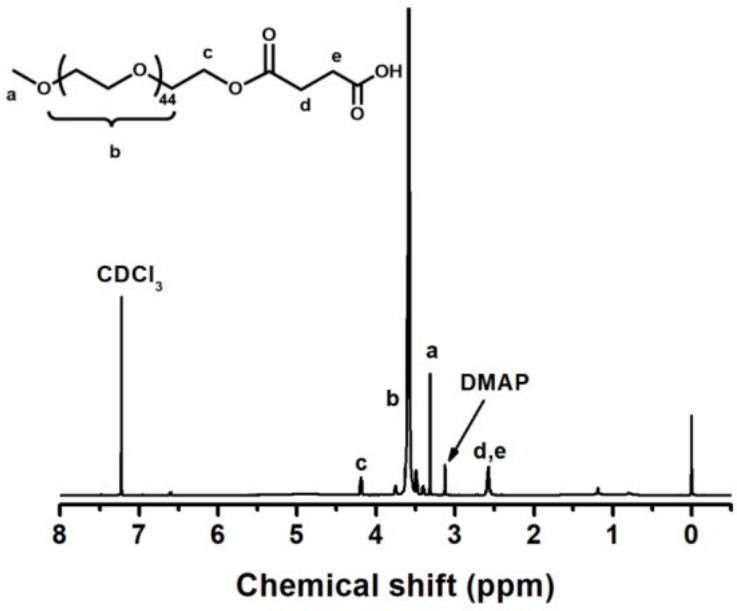


Figure S7. ^{1}H NMR spectrum of PEG-COOH

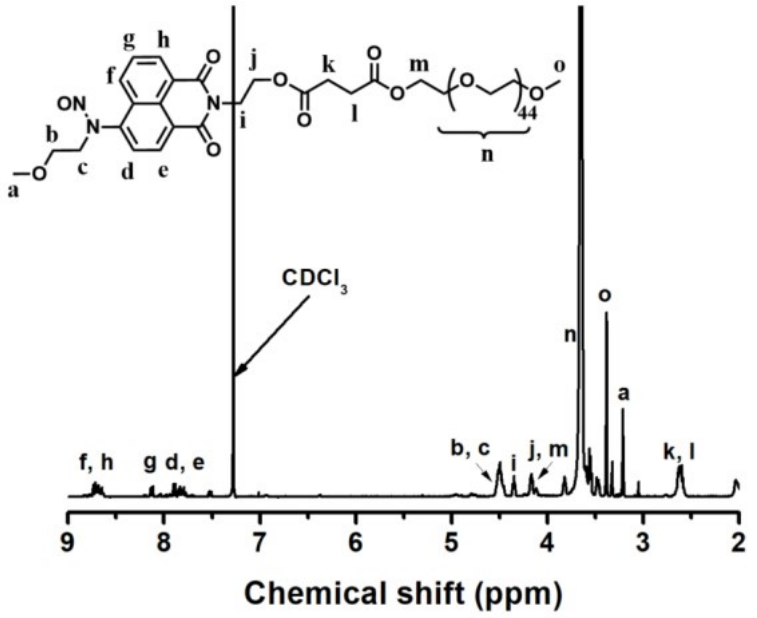


Figure S8. ^{1}H NMR spectrum of PEG-NORM

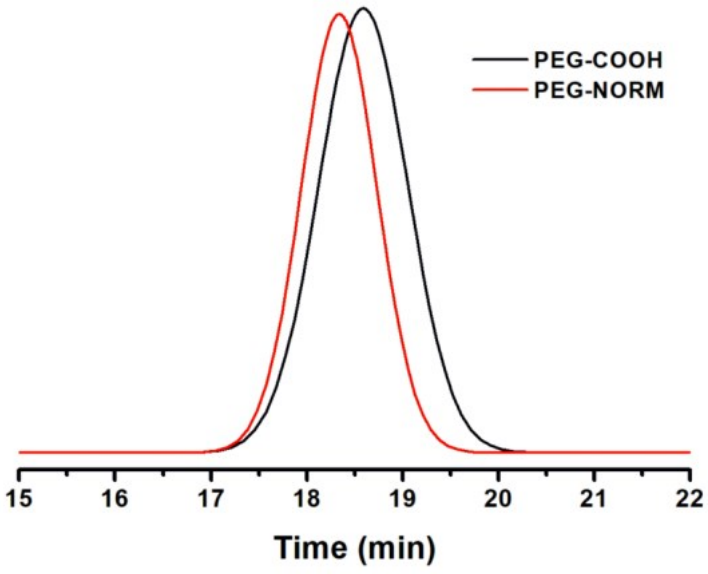


Figure S9. GPC traces of PEG-COOH and PEG-NORM

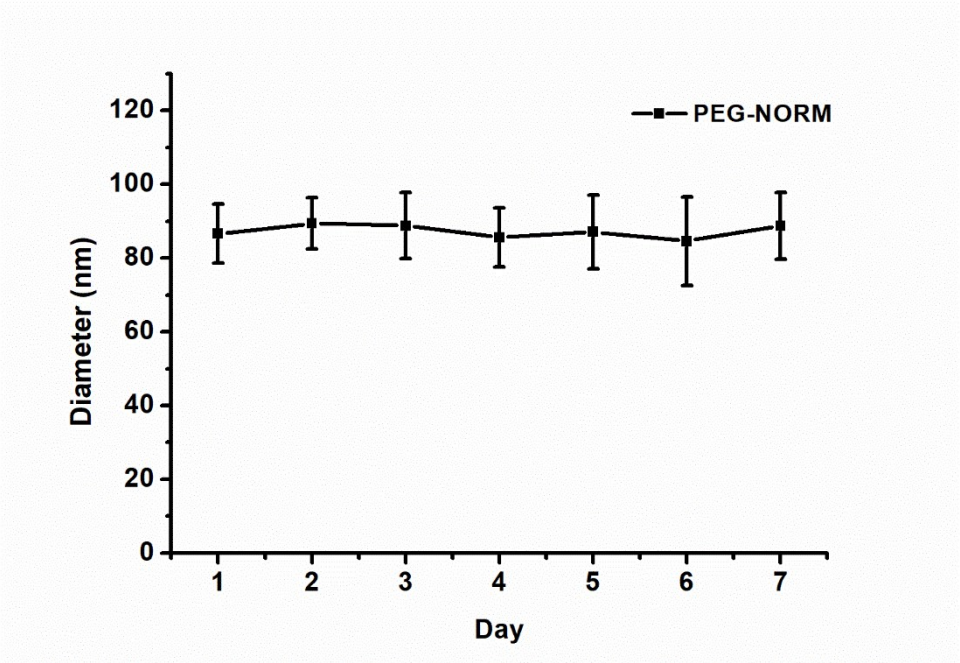


Figure S10. Hydrodynamic diameter variation of PEG-NORM nanoparticles for 7 days.

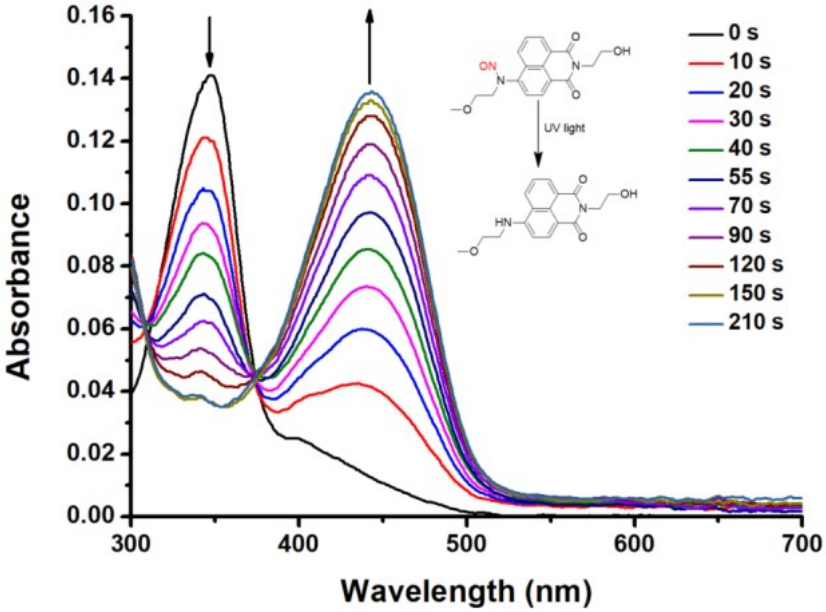


Figure S11. UV-vis absorption spectra of the NORM under UV light irradiation for different times

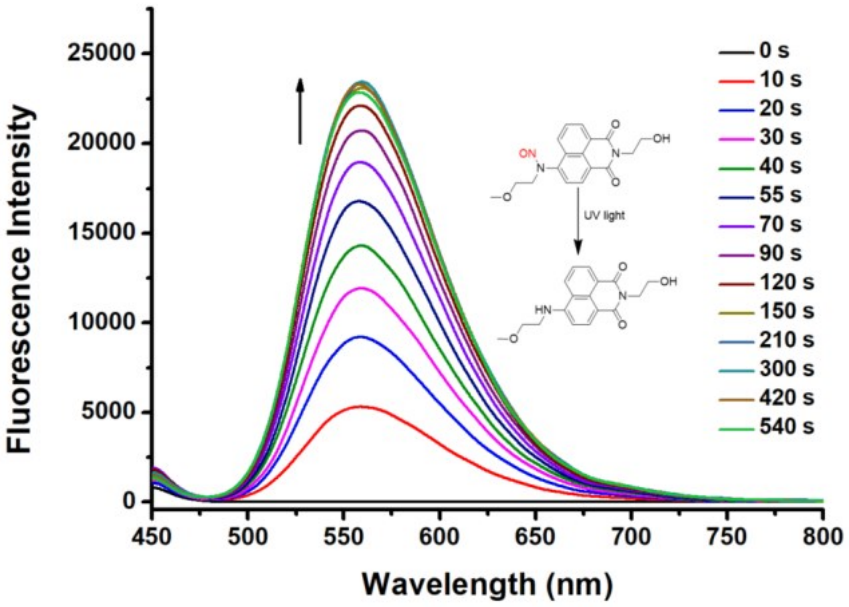


Figure S12. Fluorescence spectra of NORM under UV light irradiation for different times (λ_{ex} = 445 nm)

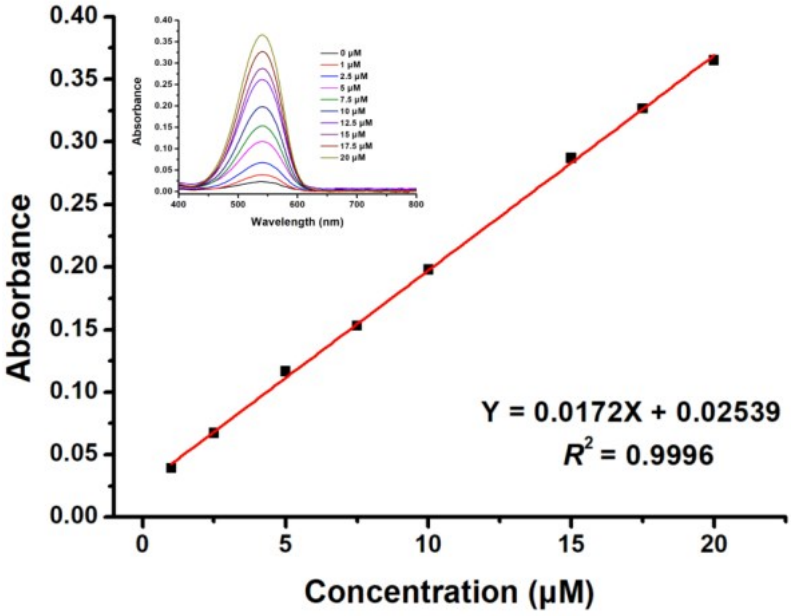


Figure S13. The standard curve of griess assay and the insert is corresponding absorption spectra with different concentrations of sodium nitrite from 1 μM - 20 μM

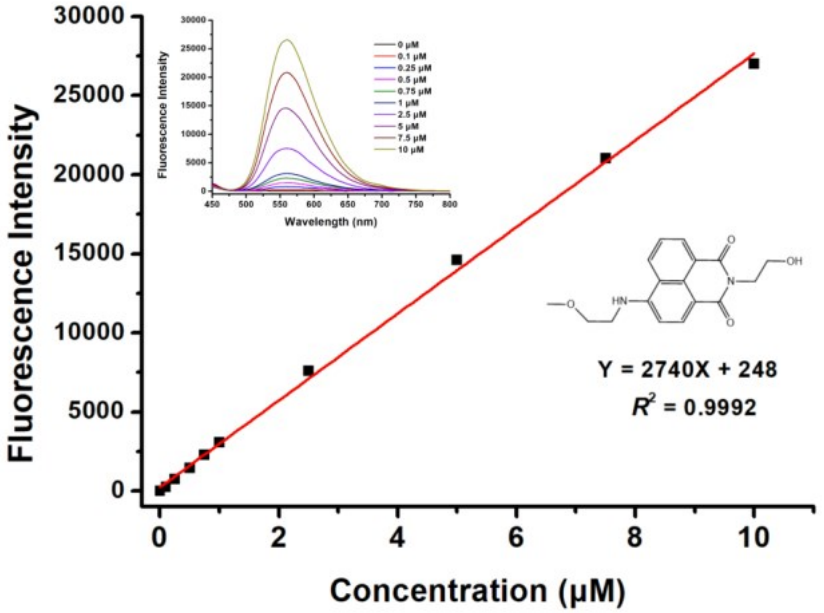


Figure S14. The standard curve of self-calibration and the insert is corresponding fluorescence spectra with different concentrations of MOEANA-OH from 0 μM - 10 μM

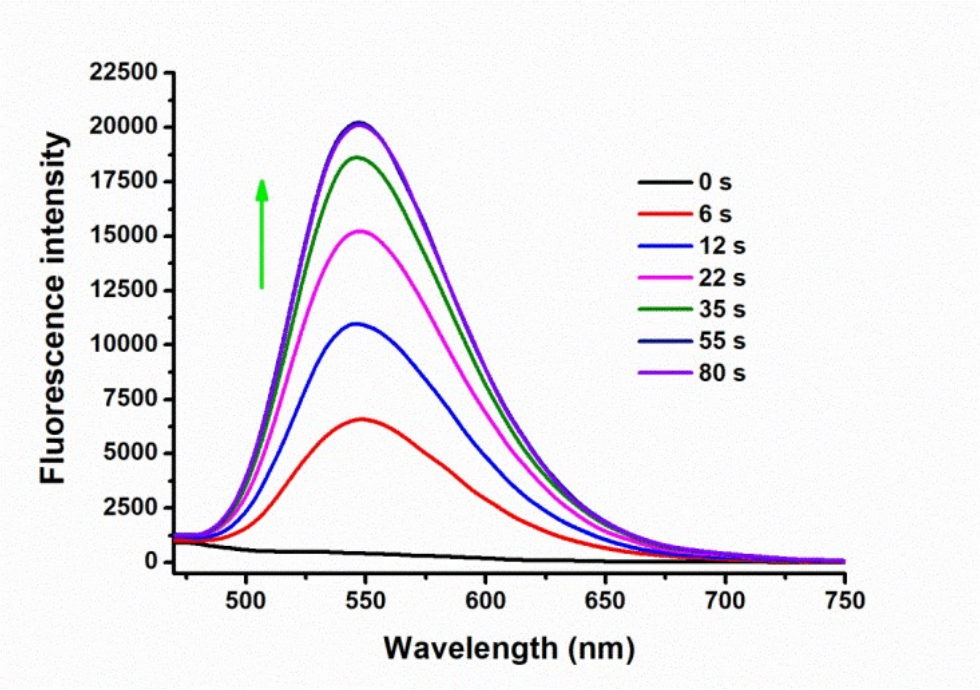


Figure S15. The fluorescence spectra of self-calibration to detect the released NO from the PEG-NORM nanoparticles irradiated with UV light with different times.

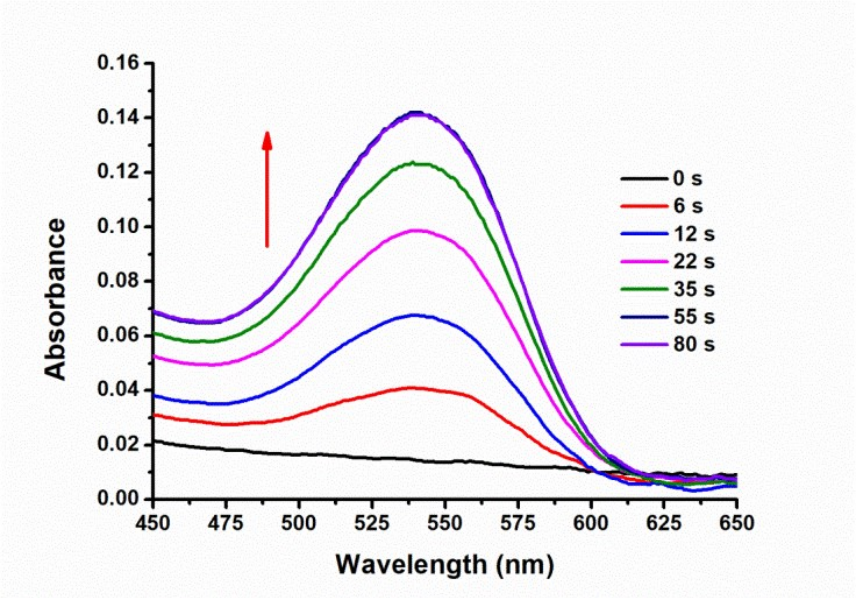


Figure S16. The UV-vis absorption spectra of Griess assay to detect the released NO from the PEG-NORM nanoparticles irradiated with UV light with different times.

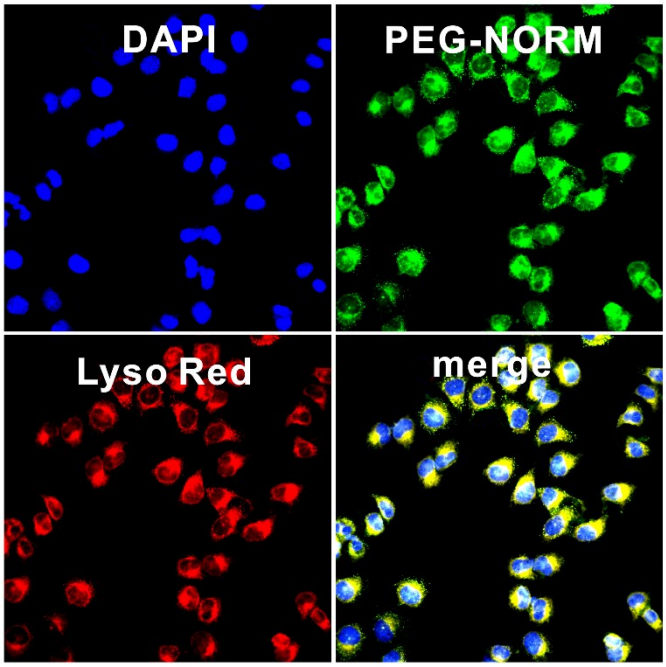


Figure S17. Cell localization assay. CLSM images of A549 cells incubated with PEG-NORM nanoparticles for 24 h. The cell nuclei were stained by DAPI with blue emission and the lysosomes were stained by Lyso-Tracker Red with red emission. The green emission represented PEG-NORM nanoparticles irradiated with UV light for 45 s.

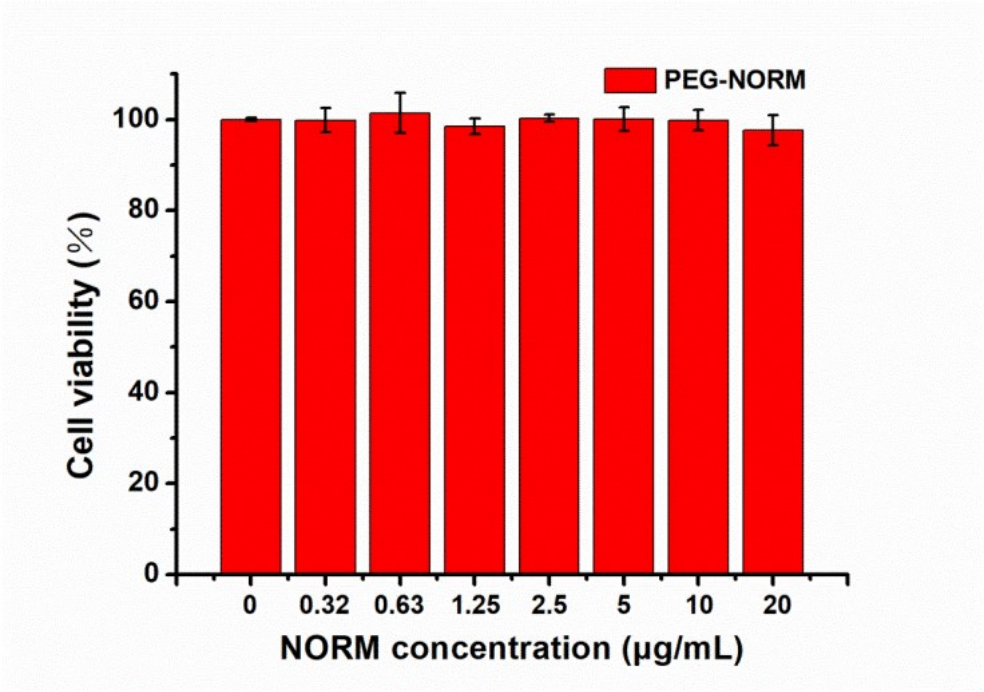


Figure S18. Biocompatibility of the PEG-NORM nanoparticles without UV light irradiation.

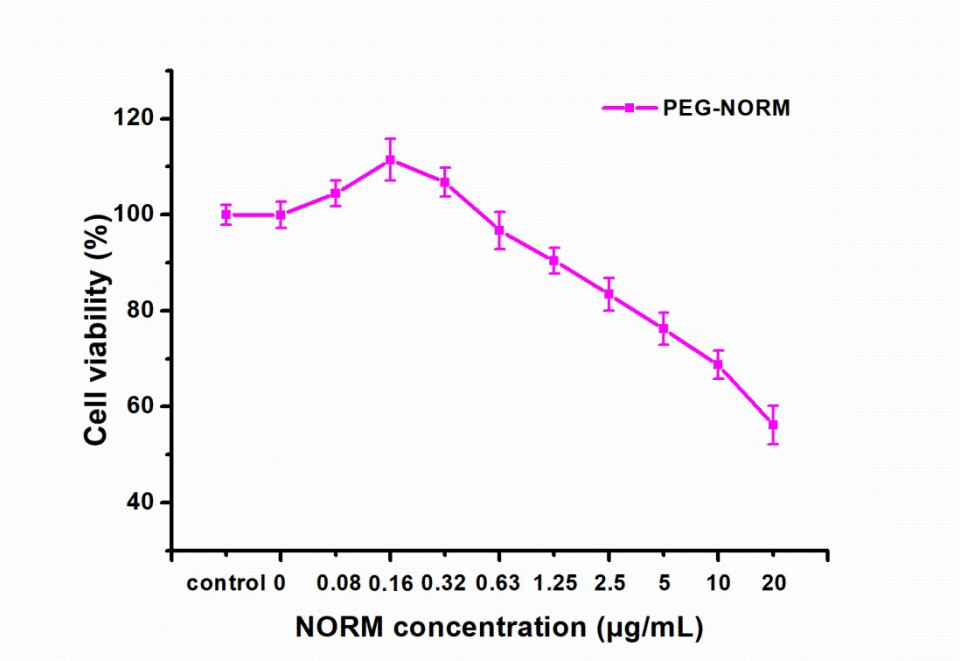


Figure S19. Cellular manipulation against L-02 cells by the PEG-NORM nanoparticles, irradiated with UV light (130 mW cm^{-2}) for 45 s, detected by MTT assay.

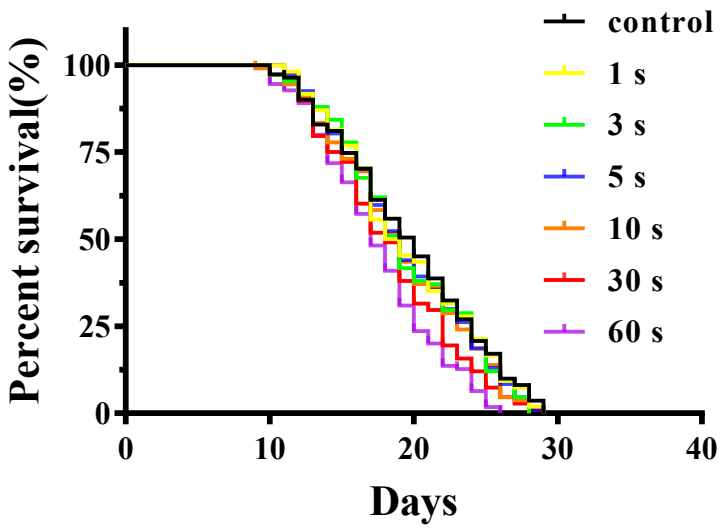


Figure S20. The influence of only UV-irradiation on the life span of worms (0 s, 1 s, 3 s, 5 s, 10 s, 30 s, 60 s, respectively).

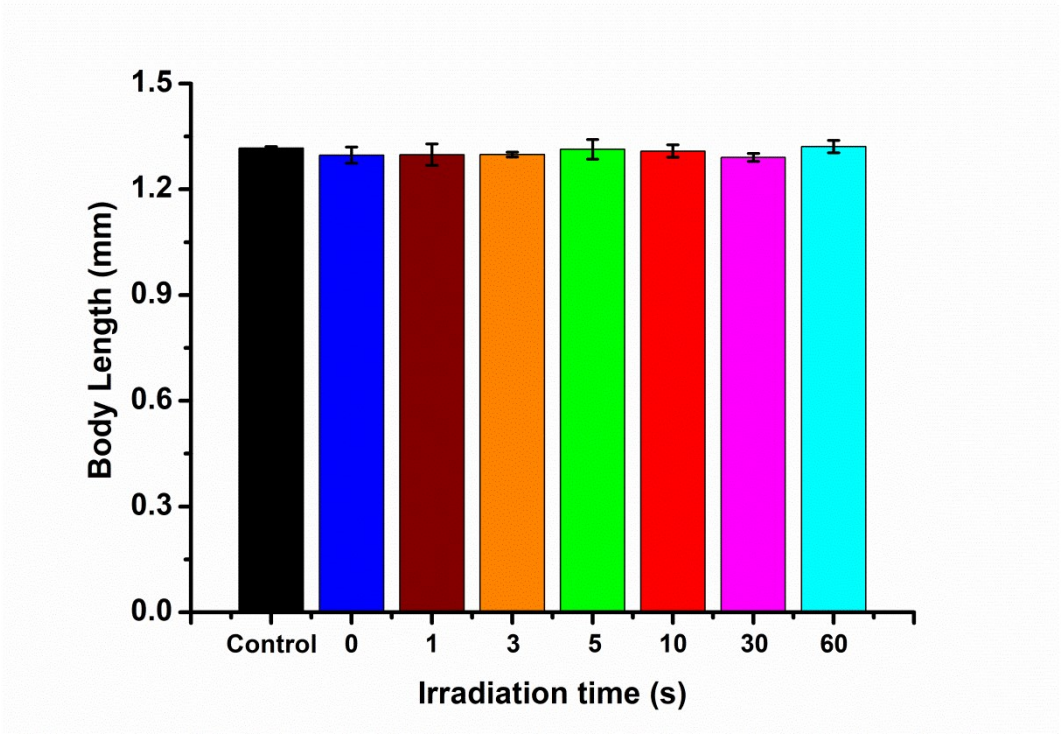


Figure S21. Body length of worms exposed to PEG-NORM nanoparticles, irradiated with UV light (130 mW cm^{-2}) for 0 s, 1 s, 3 s, 5 s, 10 s, 30 s, 60 s.

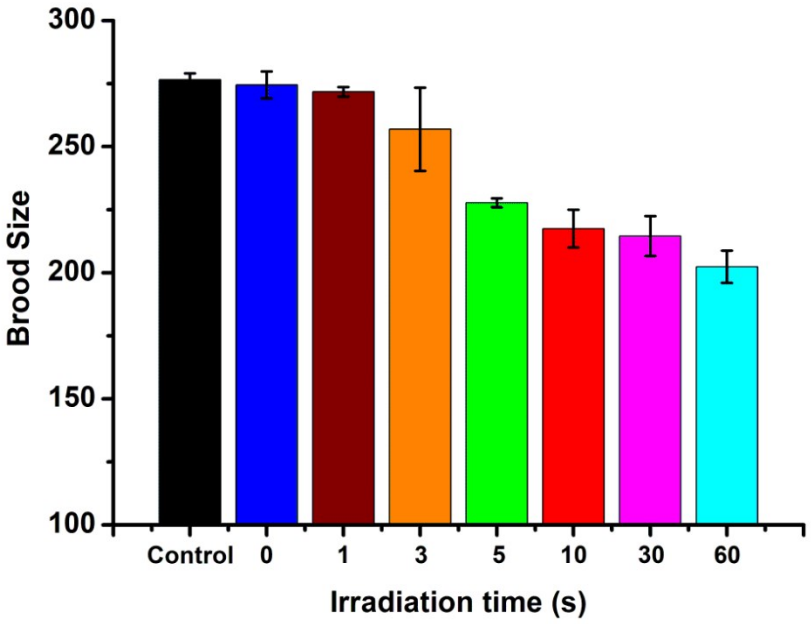


Figure S22. Brood size of PEG-NORM nanoparticles incubated worms irradiated with UV light (130 mW cm^{-2}) for 0 s, 1 s, 3 s, 5 s, 10 s, 30 s or 60 s.

Table S1. The statistical analysis on the data of lifespan assay of *C. elegans* only treated UV light. P values were calculated by ANOVA analysis, compared with the control group.

Groups	control	UV 1 s	UV 3 s	UV 5 s	UV 10 s	UV 30 s	UV 60 s
Mean lifespan 1 (d)	19.22	19.33	19.25	19.33	19.31	19.19	17.86
Mean lifespan 2 (d)	20.22	19.94	19.72	19.81	19.53	18.53	17.81
Mean lifespan 3 (d)	19.33	19.25	19.14	18.89	18.33	17.42	16.64
mean lifespan (d)	19.59	19.51	19.37	19.34	19.06	18.38	17.44
±SD	0.45	0.31	0.25	0.37	0.52	0.73	0.56
p		0.899	0.736	0.705	0.415	0.066	0.001

Table S2. The statistical analysis on the data of lifespan assay of *C. elegans* treated with PEG-NORM nanoparticles and UV light. P values were calculated by ANOVA analysis, compared with the control group.

Groups	control	NPs+UV 0 s	NPs+UV 1 s	NPs+UV 3 s	NPs+UV 5 s	NPs+UV 10 s	NPs+UV 30 s	NPs+UV 60 s
Mean lifespan 1 (d)	18.25	18.19	19.58	20.67	18.08	17.81	16.28	16.25
Mean lifespan 2 (d)	19.00	18.64	19.69	21.06	18.25	17.67	16.33	15.81
Mean lifespan 3 (d)	19.36	19.56	20.69	21.67	19.03	17.92	16.58	15.56
mean lifespan (d)	18.87	18.80	19.99	21.13	18.45	17.80	16.40	15.87
±SD	0.46	0.57	0.50	0.41	0.41	0.10	0.13	0.29
p		0.922	0.140	0.003	0.583	0.157	0.001	0.000